THE ISOLATION OF SPIROPLASMAS FROM MOSQUITOES IN MACON COUNTY, ALABAMA[1,2]

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ABSTRACT. During the summer months of 1985, 1,298 adult mosquitoes comprising 21 species and 7 genera were collected in Macon County, Alabama. Mosquitoes were collected from four sections of the county with CO_2-baited light traps. Spiroplasma cultures were isolated from two pools of 24 and 25 *Aedes fulvus pallens*, one pool of 22 *Anopheles punctipennis* and one pool of 7 *Culex nigripalpus*. Electron microscopic studies of the isolates revealed helical, wall-less cells.

INTRODUCTION

Spiroplasmas were first recognized as wall-free procaryotes in 1972 (Davis et al. 1972). The motility of these procaryotes was recognized by Davis and Worley (1973); who gave these organisms the trivial name "spiroplasma". All spiroplasmas are known to be associated with arthropods at some stage of their life cycles (Clark 1982, 1984). Several leafhopper species transmit spiroplasmas from plant to plant (Oldfield et al. 1976). Some arthropods are also capable of transmitting spiroplasmas transovarially (Williamson and Poulson 1979). To date, spiroplasmas have been isolated *in vitro* from ticks (Clark 1964; Tully et al. 1977, 1981), honeybees (Clark 1977), homopterous insects (Lee et al. 1973, Oldfield et al. 1976), tabanid flies (Clark et al. 1984), coleopterous insects (Clark 1982) and observed *in situ* in fruit flies (*Drosophila* sp.) (Williamson and Poulson 1979). The *Drosophila* organism was later cultivated in a cell line and after adaptation was cultivated in a cell-free medium (Hackett et al. 1986).

The first isolation of a spiroplasma from a mosquito was reported by Slaff and Chen (1982) who isolated these organisms from a pool of 30 *Aedes sollicitans* (Walker) taken from a study site in West Creek, New Jersey. Serological test of the isolate indicated that this spiroplasma represented an undescribed serotype. Recently, a group of French workers have reported six spiroplasma isolations from *Aedes* mosquitoes collected in the Alps in France (Chastel et al. 1985). Darsie and Ward (1981) listed 55 mosquito species representing 10 genera for the state of Alabama; thirty of these species, representing 9 genera are found in Macon County (W. E. Johnson, Jr. and L. Harrell, unpublished data). This study was designed to determine if spiroplasma could be isolated from mosquitoes in Macon County, Alabama.

MATERIALS AND METHODS

Media. Three media, which were similar to Liao and Chen's formulation (1977), were used in this study. The basal ingredients for all media were PPLO broth base, sucrose or dextrose and serum. Medium 1 (M-1) was prepared with 1.5 gm (w/v) PPLO broth base (Difco Laboratories, Detroit, MI), 8 gm (w/v) sucrose, 0.002 gm (w/v) phenol red and 10 ml (v/v) horse serum (Hyclone Laboratories, Logan, UT) as described previously by Davis et al. (1981). Medium 2 (M-2) (C. Stevens, unpublished), contained 1.5 gm (w/v) PPLO broth base, 14 gm (w/v) sucrose, 0.002 gm (w/v) phenol red and 10 ml (v/v) fetal calf serum. Medium 3 (M-3) (Liao and Chen 1979) contained 1 gm (w/v) dextrose, 2 gm PPLO broth base, 0.002 gm (w/v) phenol red and 20 ml (v/v) horse serum. Solid medium was prepared with 1 gm of Noble agar (Difco Laboratories). All ingredients except serum were dissolved in distilled water to give total volumes of 90, 90, 80 ml of M-1, M-2 and M-3, respectively. The pH was adjusted to 7.6. The basal media were then sterilized at 121°C for 20 minutes. Sterile fetal calf or horse serum was added aseptically to the cooled media.

Mosquito collection methods. Early in the study, the county was divided into four quadrants. Adult mosquitoes were collected at different sites near creeks, swamps and woodland areas in Macon County, Alabama.

Live mosquitoes were collected using battery operated Center for Disease Control (CDC) miniature light traps (Sudia and Chamberlain 1962) baited with dry ice. Collections were made from the collection sites twice weekly. Live female mosquitoes were collected, sorted, identified and

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pooled in the laboratory utilizing a dissecting microscope. Pools contained about 1-30 female mosquitoes of each species.

Cultivation and isolation. The pooled mosquitoes were ground in 3 ml of M-2 medium using a mortar and pestle. The homogenate was filtered by sterile hypodermic syringe through a 0.45 micron filter (Gelman Scientific Inc., Ann Arbor, MI) into sterile culture tubes, according to Slaff and Chen's (1982) technique for spiroplasma isolation. Aliquots (0.2 ml) of filtrates were inoculated in 2 ml of M-1, M-2 and M-3 media in duplicate sterile culture tubes. The diluted primary cultures and original filtrates were incubated at 30°C. The cultures were examined every day for 21 days. Spiroplasma cultures were first indicated by acidification of the media and confirmed by dark-field microscopy. Isolates were serially subcultured in M-1, M-2 and M-3 media. Solid medium was inoculated with 0.1 ml of a serial diluted culture. Cultures on agar plates were incubated at 30°C in plastic bags in a moist environment. These cultures were examined each day for growth of colonies.

Morphology and ultrastructure. Cultures of all spiroplasma isolates were routinely monitored by dark-field microscopy. Procedures for negative staining and ultrastructure electron microscopy (Cole 1983) have been described previously.

RESULTS

Mosquito collection and isolation of spiroplasmas. During the period of June to August of 1985, 1,298 mosquitoes were collected. All isolations were obtained in June. Two pools collected on June 6 and June 12, 1985 consisting of 49 females of *Aedes fulvus pallens* Ross and pools collected on June 2 and June 26 consisting of 22 and 7 *Anopheles punctipennis* (Say) and *Culex nigripalpus* Theobald, respectively, were positive (Table 1).

Four spiroplasma strains were isolated from three species of mosquitoes. One hundred percent isolation was obtained from pools of *Ae. fulvus pallens* collected June 6 (AEF-1) and June 12, 1985 (AEF-2) in three different media replicated two times each. Primary isolation rates of 67% and 17% were obtained from *Ae. punctipennis* (ANP) and *Cx. nigripalpus* (CXN), collected on June 2 and June 26, respectively. Success of all primary isolations was evident within 5 days.

Culture characteristics. The AEF-1, AEF-2, ANP and CXN spiroplasma isolates grew readily in M-1, M-2 and M-3 media maintained at 30°C. The AEF-1 and CXN spiroplasmas were extremely fast growing organisms as evidenced by the presence of turbidity and acidification of the medium within 17 hours.

Growth of spiroplasmas on M-2 agar was evidence by the presence of three colony types. The first type of colony, formed by ANP and AEF-2 was granular and diffuse, and formed satellite colonies from the initial site of the colony development (Fig. 1A). The second type of colonies were minute granular with discrete centers. These colonies were formed by the isolate AEF-

Table 1. Distribution of mosquitoes collected in 1985 and the pools positive for spiroplasma.

Species	Number of mosquitoes collected				No. of pools	
	June	July	August	Total	Neg.	Pos.
Ae. aegypti	4	4	7	15	8	—
Ae. atlanticus	4	0	5	9	4	—
Ae. canadensis canadesis	3	0	0	3	1	—
Ae. fulvus pallens	57	5	0	3	1	2
Ae. sticticus	24	8	2	34	10	—
Ae. triseriatus	22	2	0	24	0	—
Ae. vexans	43	20	6	69	14	—
An. crucians	64	68	19	151	19	—
An. punctipennis	70	55	4	129	14	1
An. quadrimaculatus	95	69	26	190	19	—
Cq. perturbans	8	19	5	32	9	—
Cs. melanura	1	0	0	1	1	—
Cx. erraticus	10	88	293	391	22	—
Cx. nigripalpus	7	0	1	8	1	1
Cx. quinquefaciatus	11	4	12	27	9	—
Cx. restuans	3	0	0	3	1	—
Ps. ciliata	1	1	0	2	2	—
Ps. columbiae	6	4	87	97	8	—
Ps. ferox	26	5	5	36	10	—
Ur. sapphirina	0	10	5	15	2	—
Total				1,298	160	4

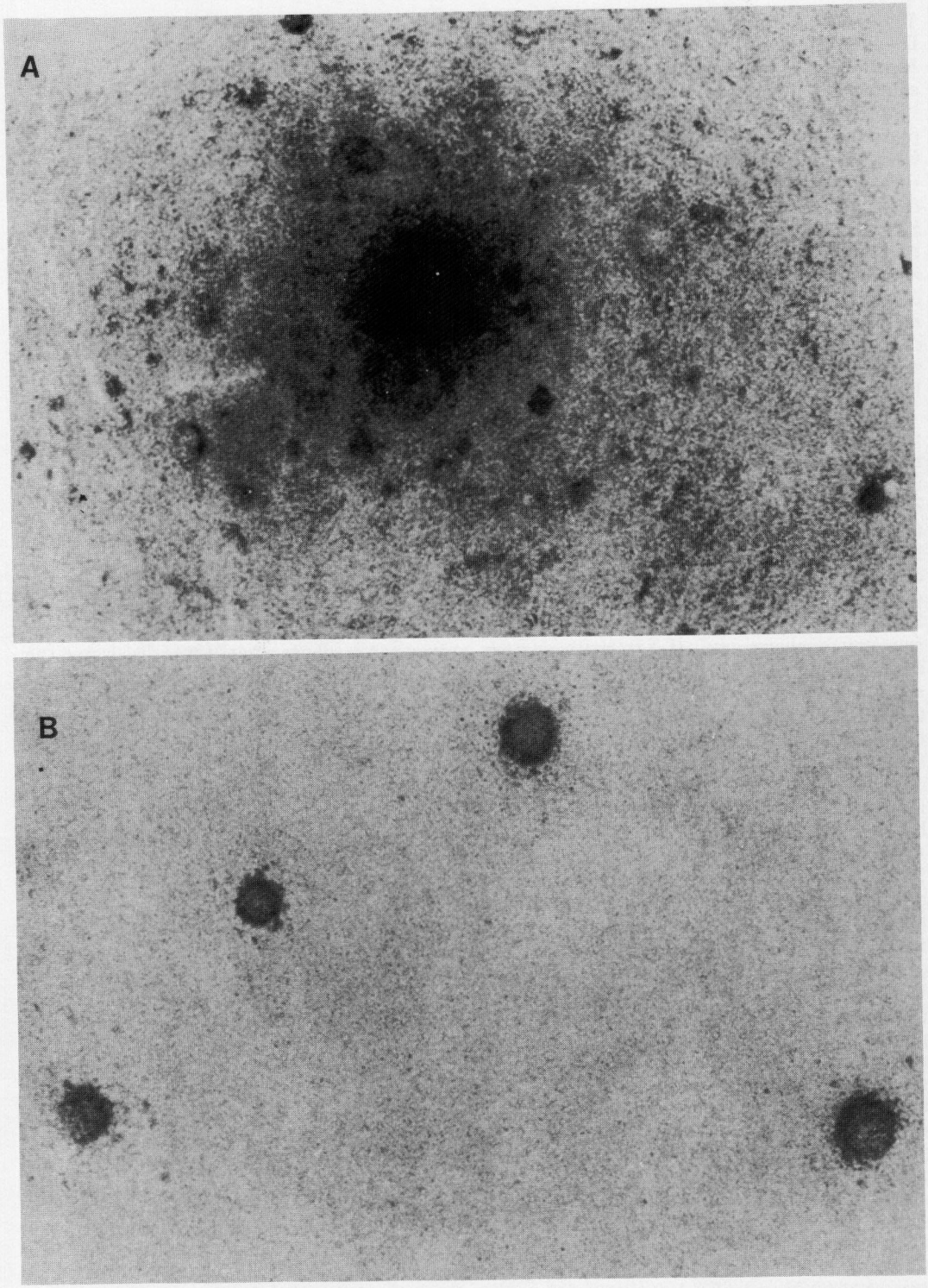

Fig. 1. The colony formation of ANP isolate from *Anopheles punctipennis* on agar (A) showing satellite growth around central zone and (B) colonies of AEF-1 isolate from *Aedes fulvus pallens* showing central core colonies (phase-contrast microscopy 100X).

1 (Fig. 1B). A third colony type was similar to the first type, but the colonies were larger and highly diffuse. Growth of these isolates on M-2 solid medium required 3 to 5 days of incubation.

Morphological characterization. The spiroplasma cells of strains AEF-1, AEF-2, ANP and CXN, as seen by dark-field microscopic observation, were helical filaments exhibiting the flexional and rotational motility typical of organisms in the genus *Spiroplasma* (Cole et al. 1973, Tully and Whitcomb 1981). Transmission electron microscopy of negatively stained cells of all four isolates revealed cells with helical morphology (Fig. 2A). Ultrathin sections of these organisms revealed the lack of a cell wall, and trilaminar unit membrane typical for mollicutes (Fig. 2B) (Whitcomb and Tully 1983).

Serological characterization. The four spiroplasma strains were examined by one-way serological deformation tests (DF) (Williamson et al. 1979). Cells of each strain were mixed with diluted sera of spiroplasma subgroups I-1 through I-7 (Whitcomb et al. 1983); subgroup I-8 (Saillard et al. 1987); groups III-XI (Whitcomb et al. 1983) and putative groups XII-XXIII (Tully et al. 1987, Williamson et al. 1988). Strains ANP and AEF-2 failed to react with sera from any numbered group (I-XI). However, each of these strains reacted with sera prepared against the CB-1 and CC-1 strains derived by Clark and his associates from cantharid beetles (Clark et al. 1987), and the Ar-1357 strain obtained by Chastel and his associates from mosquitoes in the French Alps. The intermediate levels of crossing of strains ANP and AEF-2 between alpine mosquito isolates and North American cantharid beetle strains establishes, for the first time, links among these ecologically diverse groups of strain clusters. This assemblage of diverse groups will be given the designation "group XVI" in the forthcoming revision of spiroplasma group classification (Tully et al. 1987). Isolates CXN and AEF-1 proved difficult to serotype by DF serology because their morphologies became rapidly distorted during normal growth in the M1D medium employed for the tests. However, the poorly helical forms of both of these strains shared broad patterns of deformation with sera directed against several group I subgroups, and numerous other groups including X, XI, XIII, XVII, XIX and XXI. Although this spectrum of crossing is reminiscent of the broad spectrum of nonspecific one-way crosses observed with PUP-1 lampyrid beetle strain (Clark et al. 1987), the heterologous crosses of CXN and AEF-1 antigens with anti-PUP-1 antisera had titers of only 80 and 320, respectively, compared to a homologous PUP-1 vs. anti-PUP-1 titer of 5120. Hence CXN and AEF-1 strains are at most, distantly related serovars of the PUP-1 strain group designated as group XIX by Tully et al. (1987). Alternatively, these strains may prove to be representatives of a previously recognized spiroplasma group. Only intense reciprocal serological studies, such as those proposed recently by Whitcomb et al. (1987), can resolve the taxonomic status of strains CXN and AEF-1.

DISCUSSION

The present study is the third report on the isolation of spiroplasmas from mosquitoes and the first for *Ae. fulvus pallens*, *An. punctipennis* and *Cx. nigripalpus*. Strains AEF-1, AEF-2, ANP and CXN clearly belong to the genus *Spiroplasma*, as evident by their morphological and ultrastructural characteristics (Tully and Whitcomb 1981).

Since the isolations of these spiroplasmas in this study were made from blood-sucking insects that feed on a wide variety of vertebrate hosts (Gordon and Lavoipirre 1969, Tolbert and Johnson 1982), it is conceivable that these spiroplasma isolates may be potential pathogens. The concept that spiroplasma can infect vertebrates was given credence when it was discovered that *Spiroplasma mirum*, which was isolated from the rabbit tick, could induce cataract or lethal encephalitis in suckling vertebrate animals, and was virulent for the chick embryo (Clark 1964, Tully et al. 1977, Kirchhoff et al. 1981). Presently, the role of spiroplasmas in naturally occurring vertebrate disease is not known (Clark and Rorke 1979). It is not known whether blood-sucking insects can acquire spiroplasmas in nature by feeding on vertebrate hosts (Tully et al. 1977).

There is a possibility that the three species of mosquitoes may have acquired the spiroplasmas from contaminated flowers. It is generally accepted that mosquitoes make frequent contact with plants for nectar (Magnarelli 1978). Recently, Hackett et al. (1984) have isolated subgroup I-6 spiroplasmas from fall flower hosts and from the gut of nectar-feeding insects which forage on flowers.

ACKNOWLEDGMENTS

The authors acknowledge with thanks R. F. Whitcomb and E. A. Clark, Insects Pathology Laboratory, Plant Protection Intitute, Beltsville Agricultural Research Center, USDA/ARS. Beltsville, MD 20705 for their assistance as related to the serological typing of the spiroplasmas and review of the manuscript. This research was supported in Part by the National Institutes

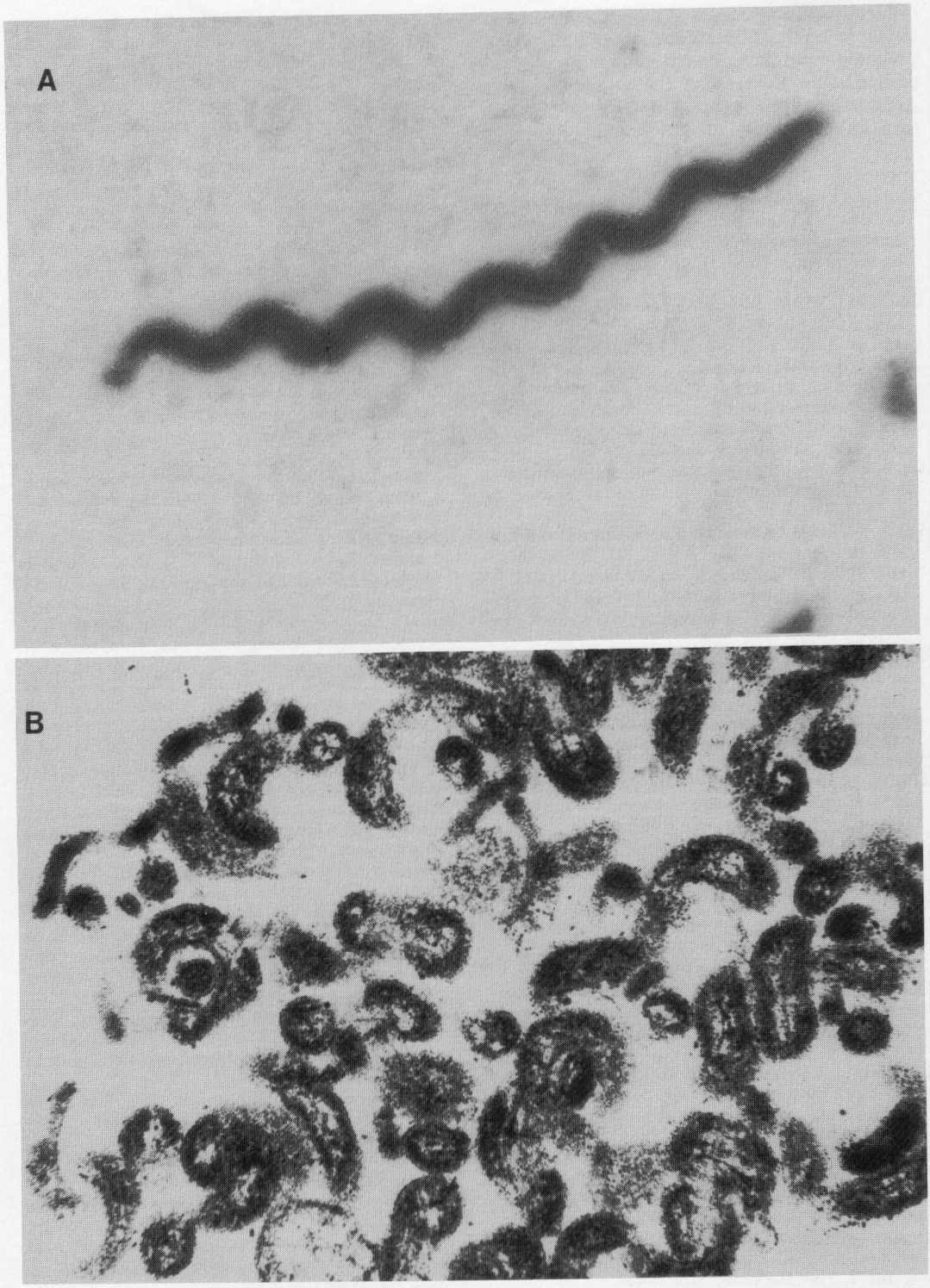

Fig. 2. (A) Transmission electron microscopy of ANP isolate from *Anopheles punctipennis* (15,000X) negatively stained with 2% phosphotungstate and (B) electron micrograph of thin section of CXN isolate from *Culex nigripalpus* (34,000X).

of Health, MBRS Grant 2-506RR08091-14 and the George Washington Carver Agricultural Experiment Station, Carver Research Foundation of Tuskegee University, USDA/CSRS Grant ALX-VC-1, FAO and the Ministry of Agriculture, Pakistan.

REFERENCES CITED

Chastel, C., B. Gilot, F. Le Goff, R. Gruffaz and M.-L. Abalain-Colloc. 1985. Isolement de spiroplasmes en France (Savoie, Alpes du Nord) a partir de moustiques du genre Aedes. C. R. Acad. Sci. Paris Ser. D. 300:216–266.

Clark, H. F. 1964. Suckling mouse cataract agent. J. Infect. Dis. 114:476–487.

Clark, H. F. and L. B. Rorke. 1979. Spiroplasmas of tick orgin and their pathogenicity, p. 155–174. *In:* R. F. Whitcomb and J. G. Tully (eds.), The Mycoplasma, vol. 3. Academic Press, New York.

Clark, T. B. 1977. *Spiroplasma* sp., a new pathogen in honey bees. J. Invert. Pathol. 29:112–113.

Clark, T. B. 1982. Spiroplasmas: diversity of arthropod reservoirs and host-parasite relationships. Science 217:57–59.

Clark, T. B. 1984. Diversity of spiroplasma host-parasite relationships. Israel J. Med. Sci. 20:995–997.

Clark, T. B., B. V. Peterson, R. F. Whitcomb, R. B. Henagar. K. J. Hackett and J. G. Tully. 1984. Spiroplasmas in the Tabanidae. Israel. J. Med. Sci. 20:1002–1005.

Clark, T. B., R. B. Henegar, L. Rosen, K. J. Hackett, R. F. Whitcomb, J. E. Lowry, C. Saillard, J. C. Vignault, J. M. Bove, J. G. Tully and D. L. Williamson. 1987. New spiroplasmas from insects and flowers: isolation, ecology and host association. Isr. J. Med. Sci. (in press).

Cole, R. M., J. G. Tully, T. J. Popkin, and J. M. Bové. 1973. Morphology, ultrastructure and bacteriophage infection of the helical mycoplasma-like organism (*Spiroplasma citri* gen. and sp. nov.) cultured form "stubborn" disease of citrus. J. Bacteriol. 115:367–386.

Cole, R. M. 1983. Transmission electron microscopy: basic techniques, p. 43–50. *In:* S. Razin and J. G. Tully (eds.), Methods in Mycoplasmology, vol. 1. Academic Press Inc., New York.

Darsie, R. F., Jr., and R. A. Ward. 1981. Identification and geographical distribution of the mosquitoes of North America north of Mexico. Mosq. Syst. Suppl. 1, 313 p.

Davis, R. E., J. F. Worley, R. F. Whitcomb, T. Ishijima and R. L. Steere. 1972. Helical filaments produced by a mycoplasma-like organism associated with corn stunt disease. Science 176:521–523.

Davis, R. E. and J. F. Worley. 1973. Spiroplasma: Motile, helical microorganisms associated with corn stunt disease. Phytopathol. 63:403–408.

Davis. R. E., I.-M. Lee and J. F. Worley. 1981. *Spiroplasma floricola*, a new species isolated from surfaces of flowers of the tulip tree *Liriodendron tulipifera* L. Int. J. Syst. Bacteriol. 31:456–464.

Gordon, R. M. and M. M. J. Lavoipirre. 1969. Entomology for students of medicine. Blackwell Seientific Publications. Oxford and Edinburg.

Hackett, K. J., T. B. Clark, A. Hicks, R. F. Whitcomb, E. Lowry and S. W. T. Batra. 1984. Occurrence and frequency of subgroup I-6 spiroplasma in arthropods associated with old fields in Maryland and Virginia. Israel. J. Med. Sci. 20:1006–1008.

Hackett, K. J., D. E. Lynn, D. L. Williamson, A. S. Ginsberg and R. F. Whitcomb. 1986. Cultivation of the *Drosophila* sex-ratio spiroplasma. Science 232:1253–1255.

Kirchhoff, H., J. Heitmann and J. Trautwein. 1981. Pathogenicity of *Spiroplasma* sp. strain SMCA in rabbit: clinical microbiological and histological aspects. Infect. Immunol. 33:292–296.

Lee, I.-M., G. Gartia, E. C. Calavan and G. H. Kaloostian. 1973. Citrus stubborn disease organism cultured from beet leafhopper. Calif. Agri. 27(11): 14–15.

Liao, C. H. and T. A. Chen. 1977. Culture of corn stunt spiroplasma in a simple medium. Phytopathol. 67:802–807.

Liao, C. H. and T. A. Chen. 1979. Cultivation of suckling mouse cataract spiroplasma in a simple medium. Abstract. Annu. Meet. Am. Soc. Microbiol. G-2:82.

Magnarelli, L. A. 1978. Nectar-feeding by female mosquitoes and its relation to follicular development and parity. J. Med. Entomol. 14:527–530.

Oldfield, G. N., G. H. Kaloostian, H. D. Pierce, E. C. Calavan, A. L. Granett and R. L. Blue. 1976. Beet leafhopper transmits citrus stubborn disease. Calif. Agric. 30:5.

Saillard, C., J. C. Vignault, J. M. Bové, A. Raie. J. G. Tully, D. L. Williamson, A. Fos, M. Garnier, A. Gadeau, P. Carle and R. F. Whitcomb. 1987. *Spiroplasma phoeniceum*, sp. nov., a new plant pathogenic species from Syria. Int. J. Syst. Bacteriol. (in press).

Slaff, M. and T. A. Chen. 1982. The isolation of a spiroplasma from *Aedes sollicitans* (Walker) in New Jersey. J. Florida Anti-Mosq. Assoc. 53:19–21.

Sudia, W. E. and R. W. Chamberlain. 1962. Battery operated light traps; an improved model. Mosq. News. 22:126–129.

Tolbert, R. H. and W. E. Johnson. 1982. Potential vectors of *Dirofilaria immitis* in Macon County, Alabama. Am. J. Vet. Med. Res. 43:2054–2056.

Tully, J. G., R. F. Whitcomb, H. F. Clark and D. L. Williamson. 1977. Pathogenic mycoplasma: cultivation and vertebrate pathogenicity of a new spiroplasma. Science 195:892–894.

Tully, J. G., D. L. Rose, C. E. Yunker, J. Cory, R. F. Whitcomb and D. L. Williamson. 1981. Helical mycoplasmas (spiroplasmas) from *Ixodes* ticks. Science 212:1043–1045.

Tully, J. G. and R. F. Whitcomb. 1981. The genus *Spiroplasma*. The Prokaryotes: A handbook on habitats, isolation, and identification of bacteria. Chapt. 169. Springer-Verlag, Berlin Heidelberg, NY.

Tully, J. G., D. L. Rose, E. A. Clark, P. Carle, J. M. Bové, R. Henagar, R. F. Whitcomb, D. E. Colflesh, D. L. Williamson. 1987. Revised group classification of the genus *Spiroplasma* (class *Mollicutes*) with proposed new groups XII through XXIII. Int. J. Syst. Bacteriol. (in press).

Whitcomb, R. F., T. B. Clark, J. G. Tully, T. A. Chen and J. M. Bové. 1983. Serological classification of spiroplasmas: current status. Yale J. Biol. Med. 56:454–459.

Whitcomb, R. F. and J. G. Tully. 1983. The family

Spiroplasmataceae and genus *Spiroplasma*, p. 781–787. *In*: N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology, 9th edition, Vol 1. Williams and Wilkins, Baltimore.

Whitcomb, R. F., J. M. Bové, T. A. Chen, J. G. Tully, and D. L. Williamson. 1987. Proposed criteria for an interim serogroup classification for members of the genus *Spiroplasma* (class *Mollicutes*). Int. J. Syst. Bacteriol. 37: (in press).

Williamson, D. L. and D. F. Poulson. 1979. Sex ratio organisms (spiroplasmas of *Drosophila*.), p. 175–208, *In*: R. F. Whitcomb and J. G. Tully, (eds), The Mycoplasmas. vol. 3. Mycoplasmas of plants and insects. Academic Press, New York.

Williamson, D. L., J. G. Tully and R. F. Whitcomb. 1979. Serological relationships of spiroplasmas as shown by combined deformation and metabolism inhibition tests. Int. J. Syst. Bacteriol. 29:345–351.

Williamson, D. L., J. G. Tully, and R. F. Whitcomb. 1988. The genus *Spiroplasma*. *In*: The Mycoplasma, vol. 5. R. F. Whitcomb and J. G. Tully (eds.) Academic Press, New York. (in press).